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14. ABSTRACT

Inflammatory breast cancer (IBC, ~5% of all breast cancers) is the most lethal form of breast cancer, presenting a 5-year survival rate that is less than half of the non-IBC patients. Remarkably, we have found that survival of IBC cells depends on histone deacetylase 6 (HDAC6) function. Here, first, we used these state-of-the-art system biology approaches to evaluate the response to ACY-1215 of a large series of breast cancer cells (sensitive and resistance) to identify critical hubs associated with resistance to HDAC6 inhibition. Through our studies we have found that STAT3 signaling is strongly upregulated in resistant cell lines upon inhibition HDAC6 suggesting an adaptative survival mechanism of the treated cells. Importantly STAT3 inhibitors (such as Ruxolitinib) already exist and can be easily translated to the clinic. Thus, our studies identified STAT3 inhibition as the prime candidate to synergistically interact with Ricolinostat. Additionally to STAT3, other pathways such as P38, TGF-β, and AKT has also emerged as MRs.

15. SUBJECT TERMS
Synergistic treatment, STAT3, IBC treatment, HDAC6.

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Progress Report 3rd year

1-Introduction

Inflammatory breast cancer (IBC, ~5% of all breast cancers) is the most lethal form of breast cancer, presenting a 5-year survival rate that is less than half of the non-IBC patients. Despite these facts, IBC remains poorly understood and systemic disease management relies exclusively on chemotherapy. Remarkably, we have found that survival of IBC cells depends on histone deacetylase 6 (HDAC6) function, whereas HDAC6 is mainly dispensable in non-IBCs[1]. Importantly, we have demonstrated that the leading HDAC6 inhibitor (Rocilinostat, Acetylon Inc.), which is being tested in clinical trials for other tumor types, inhibits the growth of IBC cells *in vitro* and *in vivo*. Our findings represent an exciting opportunity to develop novel targeted therapies for IBC patients.

2-Keywords

Inflammatory breast cancer, targeted therapy, HDAC6 inhibitor, Ricolinostat, Ruxolitinib, P38, STAT3.

3-Accomplishments

During the past period of support we have:

- *Task 1) Investigate HSP-90, DNAJB12 and MEAF6 as HDAC6 substrates that critically regulate the viability of IBC cells:* Despite its canonical roles in protein in proteostasis HDAC6[2] could act through other unrelated substrates. Through our collaboration with Acetylon, we have identified several novel putative substrates of HDAC6. HSP-90, DNAJB12 and MEAF6 were identified as the top candidates.

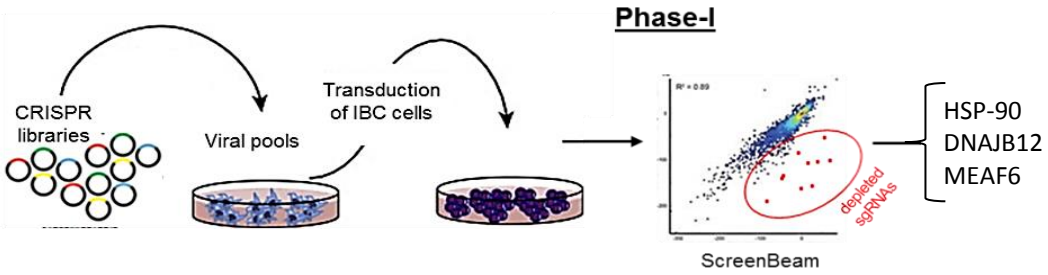


Fig 1. Representation of the completed phase-I of the genetic screen to Investigate HSP-90, DNAJB12 and MEAF6 as HDAC6 substrates that critically regulate the

Thus, we are utilizing a genetic screening strategy to investigate the involvement of these genes in the lethal phenotype induced by HDAC6 inhibitors[3,4]. We have generated a CRISPR sgRNA library containing 10 guides for each of the selected genes and an additional set of 10negative controls. This library has been used to perform genetic screens in vitro using the SUM-149 cell line. This screen validated that the three candidate genes selected scored positive for synthetic lethality in IBC.cells (Fig.1).

In order to confirm the involvement of these genes in the lethality induced by HDAC6 inhibition we performed rescue experiments. Here we overexpressed at high levels this genes in SUM-149 cells and compared the response of these lines to Ricolinostat. These studies showed that HSP-90 and MEAF6 overexpression induce resistance to HDAC6 inhibitor.

To further understand the lethality induced by inhibition of HDAC6 and its downstream targets we performed expression profiling followed by GSEA analysis of SUM cells where HDAC6 or the downstream substrates was silenced. Remarkably, these studies revealed that modulation of the chromatin remodelers CREBBP and EP300 were two critical hubs upstream to the HDAC6 signaling (Fig.2).

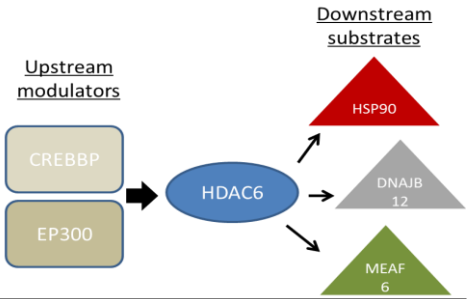


Fig 2. Models showing the upstream and downstream signaling of HDAC6.

Next, we evaluated the anticancer activity when the HDAC6 substrates HSP-90, DNAJB12 and MEAF6 are silenced utilizing RNAi (loss-of-function). Interestingly, RNAi-mediated inhibition of the individual genes compromised the fitness of the cells. Furthermore, the antiproliferative effect was stronger when the two genes were silenced at the same time (Fig 3). Importantly, when rescue experiments were performed by transducing c-DNAs expressing the corresponding genes in SUM-149 cells treated with Ricolinostat we observed 50% reduction in the anticancer activity of the drug (data not shown), suggesting that these HDAC6 targets are critical for the anticancer activity of HDAC6 inhibitors.

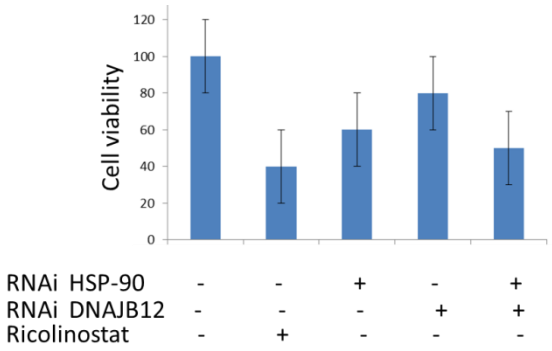


Fig 3. Silencing of HDAC6 substrates The bar graphic shows the effect in cell viability when SUM-149 cells are treated with RNAi for different genes.

- *Task 2 Design and evaluation of combination therapy with HDAC6 inhibition for IBC treatment.*

2.1-Candidate based therapy using chemotherapy plus HDAC6 inhibition.

We have pioneered the development of computational and experimental methods for identifying important hub/Master Regulators (MRs) of cancer cells. These MRs represent critical gene and pathways that modulate both cell viability and response to treatments[5,6]. Thus, these methods allow us to rationally select tumor targets as novel anticancer treatment as well as new therapeutic combinations. Here, first, we used these state-of-the-art system biology approaches to evaluate the response to ACY-1215 of a large series of breast cancer cells (sensitive and resistance) to identify critical hubs associated with resistance to HDAC6 inhibition.
Our studies have identified a series of breast cancer cell lines ((~10%) that are sensitive ((IC50>2.5uM) to HDAC6 inhibitors as well as a series (~50%) that are complete resistant (IC50>10 uM) to these treatments (Fig. 4), whit the rest of the cell models somewhere in between. Interestingly, we found that HDAC6 function was a MR only for responsive cell lines and that these lines were enriched in hormone receptor positive and Her2 positive features (Fig. 4A). Importantly, similar results were found when primary breast cancer samples were evaluated METABRIC data set[7] (Fig 4B).

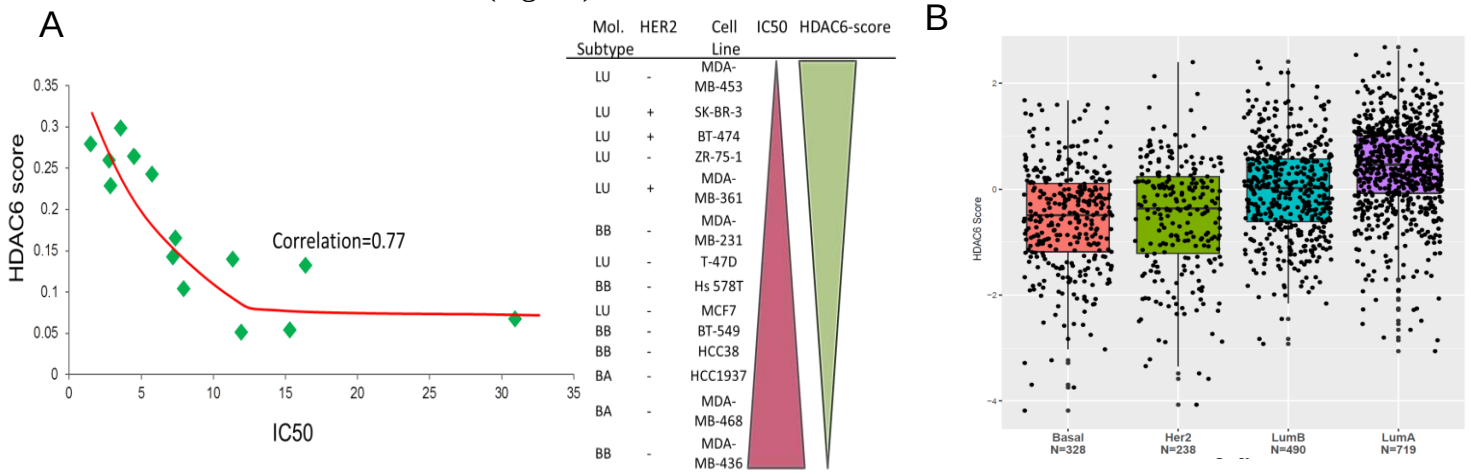


Fig. 4. MR analysis of HDAC6 response. Illustrative example of MR analysis (HDAC6 score) of cell lines and primary breast cancer samples. (A) The left panel shows the strong association between HDAC6-score and the response to the leading HDAC6 inhibitor Ricolinostat in cell lines. The right panel summarizes the result and the molecular subtype of the breast cancer lines analyzed. (B) The graphic show the HDAC6 score when the primary breast (METABRIC) samples are stratified based on molecular subtypes

Our analysis of the HDAC6 score in primary breast cancer and in cell line models have showed that HER2+ cells present high values suggesting an enhanced sensitivity to HDAC6 inhibitors. Thus, we also expanded our studies to a transgenic model where breast cancer is driven by oncogenic HER2 (FVB/N-Tg(MMTVneu)202Mul/J). We used this model to perform the same treatments described above (Fig 5). Remarkably, a significant positive response was observed. Remarkably, these studies suggest that another breast cancer types, other than IBCs can benefice of HDAC6 inhibition therapy.

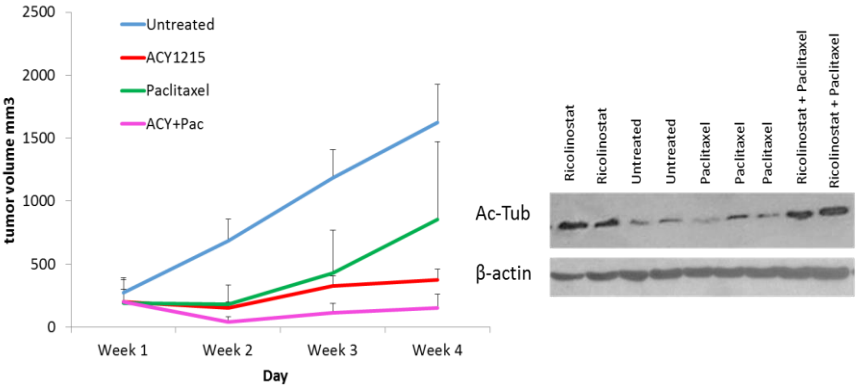
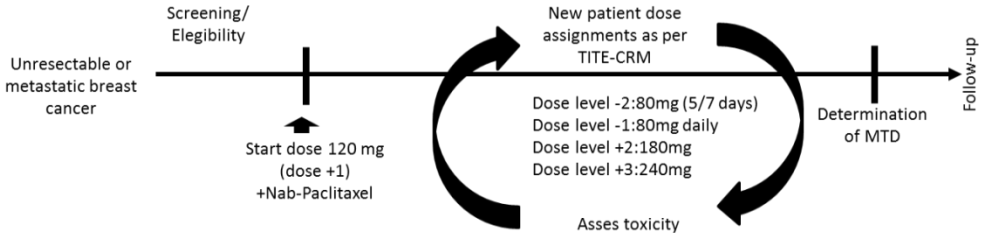


Figure 5. Anticancer activity of ricolinostat in HER2 transgenic animals. Growth of tumors emerging in the FVB/N-Tg(MMTVneu)202Mul/J model under difference treatment. ACY-1215 was administered five days per week as a single dose of 50mgr/kg. Paclitaxel was administered twice per week as a single dose of 10mgr/kg. The western blot illustrate the accumulation of Ac-tubulin in the tumors cells when the animals are dosed with Ricolinostat.

The data collected above revealed two important findings. First, a group of breast cancers responds to anticancer regiments containing HDAC6 inhibitors. Second, we can identify these cancers by using our HDAC6 score as a predictive biomarker. Thus, in collaboration with Acetylon/Celgene (pharmaceutical company that manufactures Ricolinostat) and Dr. Kevin Kalinsky, we initiated an investigator-initiated phase-Ib clinical trial (NCT02632071). ***The following data obtained from the clinical trial were collected using additional funds / resources that are not part of the DoD grant, but are included since they are complementary studies that help present the full scope of research.***



- *Trial characteristics:*

1. Subjects have confirmed metastatic adenocarcinoma of the breast; all breast cancer subtypes are allowed.
2. Minimum number of prior treatments required given standard nab-paclitaxel dosing
   -If HER2 negative: none
   -If HER2 positive: two prior regimens containing HER2 targeted therapies in the inoperable locally advanced setting. No maximum number of prior treatments in the metastatic setting.
3. ECOG performance status of 0–1 and recovered from toxic effects of all prior therapy to grade 1 or less.
4. Women and men of all races and ethnic groups are eligible for this trial. Age >18 years.
5. Study design (adaptive phase-1b):
   -Ricolinostat/ACY1215 dosing 80mg, 120mg, 180mg, 240mg PO daily on days 1–21 in a 28-day cycle.
   -Nab-paclitaxel (Abraxane) at 100 mg/m2 30 minute IV infusion on days 1, 8, and 15 in a 28-day cycle.

-*Trial results:* While this is a non-randomized phase Ib trial with the main goal of determining the maximum tolerated dose (MTD) and evaluating the safety and tolerability of Ricolinostat/ACY1215 with nab-paclitaxel, it also has the secondary goal of investigating the correlation of the HDAC6 score with patient (pt) response. Between 3/2016 and 2/2018, 17 patients were accrued; 16 were evaluable. Of evaluable pts, the median age was 57.5 (range: 41-78), 3 were TNBCs, and 13 (HR)+/HER2-. The mean number of prior lines was 4 (range: 0-9). The first patient started at 120 mg, the second at 180 mg, and the rest at 240 mg. No dose limiting toxicities were seen, and MTD was not reached. Grade III events related to nab-paclitaxel included neutropenia (n=1), peripheral neuropathy (n=1), and 1 grade IV neutropenia. Grade III syncope related to ACY-1215 was observed in 2 pts. In the 16 evaluable pts, the following were best responses: 1 partial response (PR), 11 stable disease (SD), and 4 progressive disease. One

patient with SD remains on treatment since 2/2018. We collected paraffin tissue of all these tumors and we have already performed mRNA-sequencing and calculate the HDAC6-scores for 9 of them. As expected based on our previous studies, a significant association was found between the HDAC6 score and the response to the treatment including Ricolinostat (Fig. 6A). Importantly, patients with high HDAC6 score had a significantly improved progression free survival (PFS) compared to low HDAC6 score (HR: 1.2-115, 6.6 months vs. 2.0 months, respectively p=0.01p=0.01) (Fig. 6B).

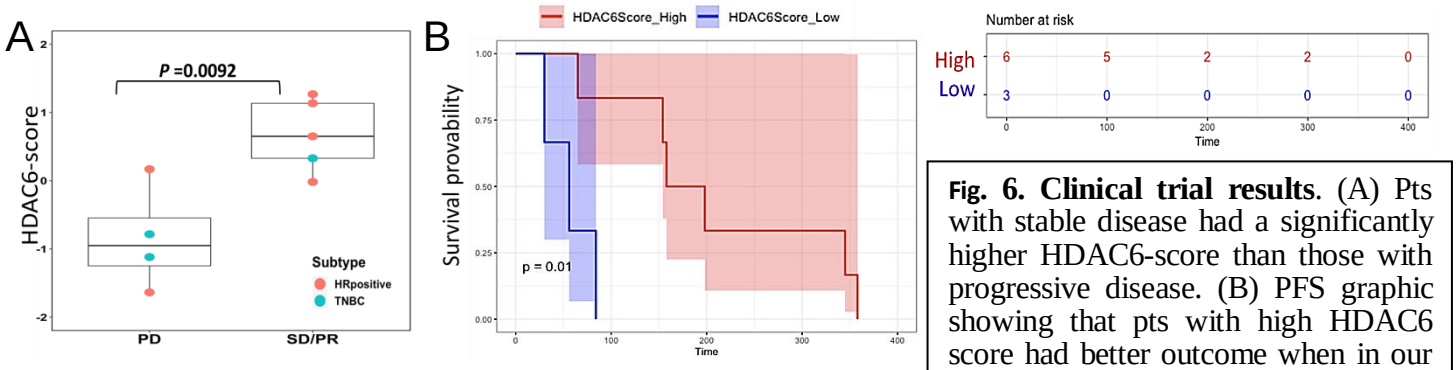


Fig. 6. Clinical trial results. (A) Pts with stable disease had a significantly higher HDAC6-score than those with progressive disease. (B) PFS graphic showing that pts with high HDAC6 score had better outcome when in our clinical trial.

-*Trial conclusions*: Ricolinostat 240mg daily is safe and tolerable with weekly nab-paclitaxel. Clinical activity has been observed, with the majority of pts demonstrating SD and PR. High HDAC6 score associates with longer PFS. HDAC6 score should be evaluated in larger trials as a predictor of response to HDAC6 inhibition.

2.2-Evaluate combinatorial regimens HDAC6 and MRs inhibitors

To investigate the mechanism of anticancer activity we performed a comparison of MR between the resistant and resistant cell lines we have found that STAT3 signaling is strongly upregulated in resistant cell lines upon inhibition HDAC6 suggesting an adaptative survival mechanism of the treated cells. Importantly stat3 inhibitors (such as ruxolitinib) already exist and can be easily translated to the clinic. Thus, our studies identified STAT3 inhibition as the prime candidate to synergistically interact with Ricolinostat. Additionally to STAT3, other pathways such as P38, TGF-β, and AKT has also emerged as MRs.

Our additional studies regarding MRs of IBC cells have also identified additional targets that enhance the activity of HDAC6 only in the presence of chemotherapy. In those studies, not covered by this grant, we have used a different computational approach to evaluate the response of IBC cells through time after exposure to Ricolinostat and chemotherapy. Interestingly those studies have suggested that proteasome inhibitors (Bortezomib), as well as mTOR inhibitors (Rapamycin), may have also synergistic anticancer activity when combined with HDAC6 inhibitors. These targets are not overlapping with the ones described above and may also expand our repertoire of putative novel targets. However, those will not be investigated under this grant funding which will be focused to the four inhibitors described above.

In order to evaluate the synergistic activity of some of the identified hubs with HDAC6 inhibitors we have evaluated combinatorial therapies using specific inhibitor and Ricolinostat (Fig.7). Multiple inhibitors for the identified pathways are available. Based on their reported selectivity, safety profile and anticancer activity we have selected:

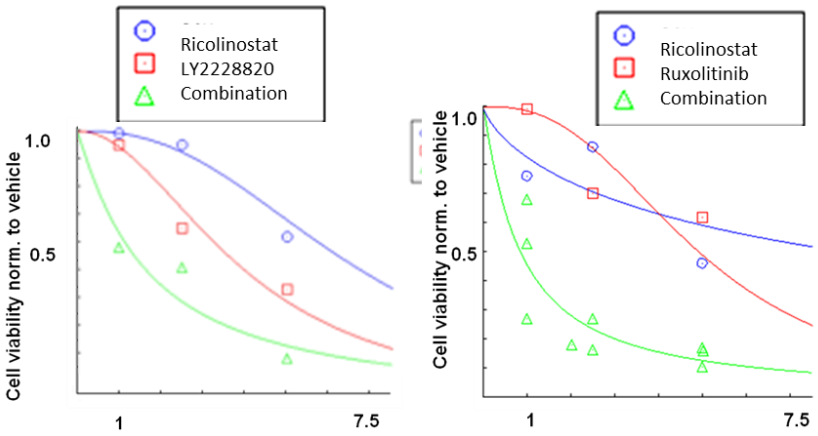
-Ruxolitinib for STAT3 modulation

- LY2228820 for P38 modulation

- LY2109761 for TGF-Beta modulation

- AT7867 for AKT-modulation

Figure 7. Synergistic activity of Ricolinostat and small molecule inhibitors targeting MR hubs. . The figure shows two examples of synergistic activity when Ricolinostat was combined with inhibitors targeting the identified MR in SUM-149 cells. .



Next we selected one of the top candidate (AKT-inhibition) to perform Additional validation in SUM-149, SUM-190 and IBC-3 cell lines. Fore that we decided to use Akti-1/2 compound that is a potent and selective dual Akt1 and 2 inhibitor (IC50 values are 50 and 210 nM, respectively) and has been reported to be active in vivo. As expected, these studies validated the results performed with the initial AT7867 inhibitor (Fig. 8)

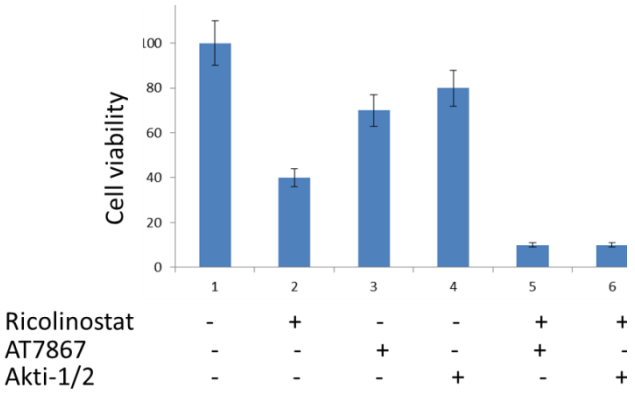


Fig 8. Silencing of HDAC6 substrates The bar graphic shows the effect in cell viability when SUM-149 cells are treated with different combinatorial treatments containing Ricolinostat.

<u>During the next period of support we will:</u>

a) **-Task1:** Evaluate the synergism anticancer activity when the HDAC6 substrates HSP-90, DNAJB12 and MEAF6 are silenced utilizing RNAi (loss-of-function in vivo.

b) **–Task2:** Investigate the top synergistic candidates for in vivo validation. Here we will evaluate the growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (when treated with small molecule inhibitors for the selected candidates plus Ricolinostat. Additionally, we will complete our studies by comparing these data with the growth inhibitory response of orthotopic xenograft mouse model of SUM-149

when combinatorial therapeutic regiments containing chemotherapy plus the small molecule inhibitor for the selected candidate.

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10.1186/1471-2105-7-S1-S7 (2006).

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4-Impact

The data that we have generated during the last years have generated two main clinically relevant findings. First we have found that HDAC6 is a master Regulator of Hormone Receptor and HER2 positive breast cancer cells and seconds that resistance to the anticancer activity of HDAC6 inhibitors is associated with activation of the STAT3 and P38 pathway. This opens the exciting opportunity of combining STAT3 inhibitors with HDAC6 inhibitors.

5-Changes/Problems

During the past period of support Erin Nekritz obtained her own independent funding to work in a different project. Thus, although she has been able to commit some time to this project her departure has generated some delays. A new research associate staff member has been recently hired to complete the project (Dr. Partha Mukhopadhyay, Biosketch and other support is attached at the end of this progress report). Dr. Mukhopadhyay has more than 15 years of research experience in the field of cancer biology including breast cancer. He will assume all the research responsibilities of Erin Nekritz.

Because of the above we are requesting a 12 months no-cost extension to complete the project.

Specific Aim 1(specified in proposal)	Timeline	Site 1	Site 2	Status
Specific Aim 1 tasks	Months			
1 – Investigate Novel Putative Targets (HSP-90, DNAJB12 and MEAF6).				
c) Generation and validation of shRNA and c-DNA library targeting the three selected genes	0-6	Erin Nekritz and Dr. Silva (MSSM)		Completed
d) Screens in vitro using the shRNA and c-DNA libraries in the SUM-149 cell line.	6-12	Erin Nekritz and Dr. Silva (MSSM)		Completed
e) Validation of shRNA/cDNA screens hits by lethality rescue experiments in SUM-149, Sum-190 and IBC3 cell lines	12-30	Erin Nekritz and Dr. Silva (MSSM)		completed
f) Synergism studies for HSP-90, DNAJB12 and MEAF6 loss/gain-of-function studies combining two genes at a time (rescue experiments combination of two at a time)	24-36	Erin Nekritz and Dr. Silva (MSSM)		COMPLETED
g) In vitro, genome-wide level studies evaluating the consequence of inhibiting HSP-90, DNAJB12 and MEAF6 in IBC cells. These studies will consist of expression profiling followed by GSEA of SUM-149, Sum-190 and IBC3 cell lines after the three candidate genes have been knock-down by RNAi.	12-24	Erin Nekritz and Dr. Silva (MSSM		Completed
h) The studies from e) will be complemented by in vivo studies in the cell line SUM-149 (25 SCID mice will be used).	24-36	Dr. Mukhopadhya y and Dr. Silva (MSSM)		IN PROGRESS
Specific Aim 2 tasks	Months			
Candidate based therapy using chemotherapy plus HDAC6 inhibition.		8		
a) Dose-response studies with ACY-1215 in 45 breast cancer cell lines	0-3	Erin Nekritz and Dr. Silva		Completed

to identify sensitive vs resistant breast cancer cells. b) Generate expression profiles in the selected resistant and sensitive cell lines in dose-response experiment with ACY-1215. c) Identify Master Regulators (MRs) that define responsive vs resistant cell lines to ACY-1215 (candidate driven studies). (Phase-I)	3-9 9-15	(MSSM Erin Nekritz and Dr. Silva (MSSM) Erin Nekritz and Dr. Silva (MSSM)	Dr. Mundi and Dr. Califano (Columbia Un.)	Completed Completed
Evaluate combinatorial regimens HDAC6 and MRs inhibitors in preclinical in vitro. (Phase-II). a) MR analysis normally yields a few dozen putative candidates. Here we will utilize compound inhibitors for five of the top-ranked candidates will be evaluated by dose-response experiment in vitro in SUM-149, SUM-190 and IBC-3 cell lines as well as the resistant cell lines previously identified. Selected candidates are: -Ruxolitinib for STAT3 modulation - LY2228820 for P38 modulation -LY2109761 for TGF-Beta modulation - AT7867 for AKT-modulation Validation of the top candidate from a) with an additional independent inhibitor in vitro in SUM-149, SUM-190 and IBC-3 cell lines.	12-24 20-36	Erin Nekritz , Dr. Silva (MSSM) Erin Nekritz and Dr. Silva (MSSM)	Dr. Mundi and Dr. Califano (Columbia Un.)	Completed COMPLETED
Evaluate combinatorial regimens HDAC6 and MRs inhibitors in preclinical in vivo. (Phase-III). a) Obtain ACURO approval for animal work b) Select the top candidate for in vivo	0-6 24-30	Erin Nekritz , Dr. Silva	Dr. Mundi and Dr. Califano	Completed

validation. Evaluation of growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (we will use 10 SCID mice) when treated with small molecule inhibitor for the selected candidate. c) Evaluation of growth inhibitory response of orthotopic xenograft mouse model of SUM-149 (we will use 15 SCID mice) when combinatorial therapeutic regiments containing chemotherapy plus the small molecule inhibitor for the selected candidate.	30-36	(MSSM) Dr. Mukhopadhyay and Dr. Silva (MSSM) Dr. Mukhopadhyay and Dr. Silva (MSSM)	(Columbia	IN PROGRESS IN PROGRESS

6- Products

Some of our findings has been presented in the following international scientific meetings:

- **San Antonio Breast Cancer Symposium 2018**: Title "Phase IB trial of ACY-1215 (Ricolinostat) combined with nab-paclitaxel in metastatic breast cancer."
- **ASCO-2019**: Title "Phase IB trial of ACY-1215 (Ricolinostat) combined with nab-paclitaxel in metastatic breast cancer (MBC).

7- Participants & other Collaborating Organizations

-Jose Silva: No Change.

-Erin Nekritz: She has been substituted by Dr. Partha Mukhopadhyay, Biosketch and other support is below.

-Prabhjot S. Mundi: No Change.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: **Partha Mukhopadhyay**
eRA COMMONS USER NAME: MUKHOPADHYAY. PARTHA

POSITION TITLE: Associate Scientist

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion (MM/YYYY)	FIELD OF STUDY
University of Calcutta (India)	B.S (Chemistry Honors)	09/1989	Chemistry
University of Calcutta (India)	M.S (Biochemistry)	12/1991	Biochemistry
Jadavpur University (India)	Ph. D (Science)	05/2002	Science
Vanderbilt University Medical Center, TN	Postdoctoral Research Fellow	08/2003	Lung cancer
UT Southwestern Medical Center, TX	Postdoctoral Researcher	09/2007	Breast cancer
University of Nebraska Medical Center, NE	Postdoc Research Associate	08/2012	Breast cancer, stem cells and reprogramming
Virginia Commonwealth University, VA Columbia University Medical Center, NY	Postdoctoral Researcher	06/2016	Breast cancer, lipid research and stem cells
Icahn School of Medicine at Mount Sinai, NY	Associate Research Scientist	06/2019	Breast cancer, metabolic imbalance and epigenetic regulation
	Associate Scientist	Present	Breast cancer, stem cells and aging

A. Personal Statement

During my postdoctoral training, I have investigated the molecular mechanism(s) associated with the development of various diseases to improve the current treatment and/or patients' survival. Radiation, the first line of treatment for lung cancer patients, induces IL-1β that produces Cyclooxygenase-2 (COX-2). Use of high dose selective COX-2 inhibitors could cause thrombosis or renovascular hypertension in patients. I have demonstrated that IL-1β induced COX-2 expression can be inhibited by low dose of CDK2 inhibitor BMS 387032 in lung cancer cells (Mukhopadhyay et al., 2006 Cancer research PMID: 16452236). Development of drug resistance in cancer patients is a major clinical problem. Using *in vitro* cell line model system, I have identified CXCR4 as a predictor of Trastuzumab resistance. Preclinical and clinical studies currently support the potential efficacy of CXCR4 inhibitors for cancer treatment. As CXCR4 functions in normal physiology, designing of novel combination therapies with CXCR4 inhibitors will be critical for the development of low-side-effect cancer treatment. I have also demonstrated that Mucin 4 (MUC4) mediated upregulation of EGFR and Her2 confers invasive potential to triple negative breast cancer cells (Mukhopadhyay et al., 2011 BBA reviews on Cancer PMID: 21277939; Mukhopadhyay et al., PLoS One 2013 PMID: 23408941). My earlier studies on COX-2/PGE2, CXCR4/CXCL12 and EGFR/Her-2 signaling axes are critically linked to cancer stem cells (CSCs) that are currently being proposed as important target for cancer therapy. Further, I have demonstrated that the Cl66 murine breast cancer stem cells are heterogeneous and exhibit different functional properties (Mukhopadhyay et al., 2013 PLoS One PMID: 24265713) and this may also be the case for human breast cancer stem cells. I have identified that the ABCC11 (a transporter) exported sphingosine-1-phosphate (S1P) enhances $ALDH1^+$ stem/progenitor cell development and contributes toward breast cancer progression. Currently, my research is focused on defining the basic mechanisms involved in resistance to anticancer therapy in breast cancer to develop with a focus on response to In addition, I am

investigating mechanistically the intersection of aging and breast cancer. In summary, I have a demonstrated record of accomplished and productive research in an area of high relevance for cancer research.

B. Positions and Honors

Positions and Employments

1992 - 1997	Research Scholar, Vivekananda Institute of Biotechnology (India)
1997 - 1998	Visiting Fellow, National Institute of Cholera and Enteric Diseases (India)
1998 - 2002	Senior Research Fellow, National Institute of Cholera and Enteric Diseases (India)
2002 - 2003	Postdoctoral Fellow, Vanderbilt University Medical Center (Nashville, TN)
2003 - 2007	Postdoctoral Researcher, UT Southwestern Medical Center (Dallas, TX)
2008 - 2012	Postdoctoral Research Associate, University of Nebraska Medical Center (Omaha, NE)
2012 - 2013	Collaboration with Dr. Sharp to complete a manuscript (*Mukhopadhyay et al., 2013*)
2014 - 2016	Postdoctoral Fellow, Virginia Commonwealth University (Richmond, VA)
2017	Postdoctoral Associate, Stony Brook University (Stony Brook, NY)
2018 - 2019	Associate Research Scientist, Columbia University Medical Center (New York, NY)
2019 – Present	Associate Scientist, Icahn School of Medicine at Mount Sinai (New York, NY)

Other Experience and Professional Memberships

Associate member of the American Association for Cancer Research since 2002

Honors/Awards

2000: B.S. Narasingha Rao Best Poster Award (Indian Council of Medical Research)

C. Contribution to Science

High epidermal growth factor receptor (EGFR) expression on cancer cells contributes to radioresistance. In this pre-clinical study, we demonstrated that the extent of erlotinib-induced radiosensitization was proportional to EGFR expression and its autophosphorylation (Kim et al., 2005 PMID: 16955736). I served as co-investigator in all of these studies. My later publication directly addressed ionizing radiation (IR) induced COX-2 and IL-1β production, which can be suppressed by a potent CDK2 inhibitor BMS-387032 in lung cancer cells. IR is frequently used to treat lung cancer and it initiates the expression of various circulatory cytokines through a DNA damage response. Chronic inflammation is linked to the development of cancer in several organs (Weitzman *et al*., 1990 PMID: 2200535; FitzGerald *et al*., 2003 PMID: 14668809). Selective COX-2 inhibitors provide relief from pain and inflammation; however, COX-2 inhibitors could cause thrombosis or renovascular hypertension in patients (Castellone *et al*., 2005 PMID: 16293724). We demonstrated for the first time that BMS-387032, down-regulates IL-1β-induced COX-2 expression in non–small cell lung carcinoma (NSCLC) cell lines (H358 and A549) without inhibiting basal COX-2 levels (Mukhopadhyay et al., 2006 PMID: 16452236). Thus, our novel findings present an opportunity for tumor control through the selective inhibition of cytokine- and CDK2-driven COX-2 expression in certain subsets of tumors. I served as primary investigator on this study. In another study, we

identified a new link between inflammatory and growth factor signaling pathways mediated by receptor-interacting protein-1 (RIP1) and provided insight into the mechanism used by RIP1 to regulate EGFR levels (Ramnarain *et al*., 2008 PMID: 18007664).

1. Saha D*, Kim JC, Cao Q, Mukhopadhyay P, Choy H. Correlation of EGFR expression level and the degree of radiosensitizing effect of the EGFR inhibitor erlotinib (2004). 95th AACR annual meeting, Orlando, FL, March 27–31.
2. Kim JC, Ali MA, Nandi A, Mukhopadhyay P, Choy H, Cao C, Saha D. ***Indian j of biochemistry & biophysics***. 2005; 42(6):358-65. PMID: 16955736.
3. Mukhopadhyay P, Ali MA, Nandi A, Carreon P, Choy H, Saha D*. 97th AACR annual meeting (Abs# 4917), April 2006. Cancer Res October 22 2014 66 (8 Supplement) 1155.
4. Mukhopadhyay P, Ali MA, Nandi A, Carreon P, Choy H, Saha D. ***Cancer research***. 2006; 66(3):1758-66. PMID: 16452236.
5. Ramnarain DB, Paulmurugan R, Park S, Mickey BE, Asaithamby A, Saha D, Kelliher MA, Mukhopadhyay P, et al., ***Cell death and differentiation***. 2008; 15(2):344-53. PMID: 18007664.

Several mechanisms have been proposed as potential mediators of Trastuzumab resistance in breast cancer (Lan *et al*., 2005 PMID: 16382045). Using Affymetrix analysis, we identified and later validated that the upregulation of the chemokine receptor CXCR4 is a predictor of Trastuzumab resistance in $Her2^+$ breast cancer cells. We have demonstrated that siRNA mediated genetic ablation of CXCR4 in acquired Trastuzumab-resistant breast cancer cells sensitizes these cells to Trastuzumab (Tripathy *et al.,* 2006. BCRT 100:S31*;* Mukhopadhyay *et al.,* 2007*;* Tripathy *et al.,* 2007. BCRT 106:S32). Further, research is warranted to determine whether this development of resistance is due to the fact that this pooled population may harbor resistance acquired over time or may represent a selected subpopulation of cells with inherent resistance. Pre-clinical and clinical studies support the potential efficacy of CXCR4 inhibitors for cancer treatment (Duda *et al*., 2011 PMID: 21349998); however, CXCR4-targeting anticancer therapy will show potential side effects on the stem cell compartment of normal tissues due to its function in normal physiology. Thus, design of novel combination therapies with CXCR4 inhibitors will be critical for the development of low-side-effect cancer treatments. I served as the primary investigator on this study.

1. Tripathy D*, Mukhopadhyay P, Sikder K, Rosenblatt K, Story M, Verma U, Zhao Y, Ding L (2006, January). Genomic and proteomic alterations in acquired trastuzumab resistance (Abs#550861, poster discussion#309). In Breast Cancer Research and Treatment (100: S31).
2. Mukhopadhyay P*, Verma U, Story M, Ding L, Snider AM, Avila K, Tripathy D. (2007). Role of chemokine receptor CXCR4 in acquired trastuzumab resistance. Breast Program Mini-Symposium. UT Southwestern Medical Center, Dallas, TX. Feb 9, 2007 (Oral presentation).
3. Mukhopadhyay P*, Verma U, Story M, Ding L, Snider AM, Avila K, Mukhopadhyay C, Tripathy D (2007). Upregulation and targeting of chemokine receptor CXCR4 in acquired trastuzumab resistance. Cancer Research, 67(9 Supplement), 2338 (Poster presentation).
4. Tripathy D*, Mukhopadhyay P, Verma U, Mukhopadhyay C, Shelton J, Story M, Ding L (2007, December). Targeting of the chemokine receptor CXCR4 in acquired trastuzumab resistance (Poster#306). In Breast Cancer Research and Treatment (106: S32).

Although several therapeutic options targeting EGFR, PARP1, VEGFα, Src, HDAC, and MEK are being investigated in clinical trials (Fong *et al*., 2009 PMID: 19553641), the overall prognosis of patients with triple negative breast cancers (TNBCs) remains dismal owing to a lack of effective treatment. Emerging evidence suggested that mucins were associated with breast cancer pathogenesis (Mukhopadhyay et al., 2011 PMCID: PMC3230300); however, the functional role of MUC4 (mucin 4) was not fully understood in breast cancers. In one study, we have shown that MUC16 induces enhanced proliferation and anti-apoptosis in breast cancer cells (Lakshmanan *et al*., 2012 PMCID: PMC3288594). I served as co-investigator on this study. Later, we demonstrated that MUC4 promotes invasive activities of TNBC cells by

upregulating the expression of EGFR, Her 2 and Her 3 molecules and their downstream signaling (Mukhopadhyay et al., 2013 PMCID: PMC3569463). Further, detailed analyses of global alterations in gene expression using microarray support the concept that MUC4 confers oncogenic potential to MDA-MB-231 cells. In fact, a direct role of mucins, especially MUC4 has not yet been established however; an indirect role may be acquired through the stabilization of EGFR and Her2 proteins. I served as the primary investigator on this study.

1. Mukhopadhyay P*, Batra SK. Pathobiological implications of MUC4 in breast cancer. Postdoc seminar series. University of Nebraska Medical Center (DRCI 1005), Omaha, Nebraska. Oct 15, 2010 (Oral presentation).
2. Mukhopadhyay P*, Smith LM, Batra SK. MUC4-dependent mechanisms of progression and metastasis of breast cancer. Breast Cancer Training Program seminar series. UNMC, Omaha, NE. May 31, 2011 (Oral presentation).
3. Lakshmanan I, Ponnusamy MP, Das S, Chakraborty S, Haridas D, Mukhopadhyay P *et al*., ***Oncogene***. 2012; 31(7):805-17. PMID: 21785467.
4. Mukhopadhyay P, Ponnusamy MP, Pai P, Chakraborty S, Lakshmanan I, Batra SK* (Abs#P6-13). CDMRP Era of Hope 2011, Florida. August 2-5.
5. Mukhopadhyay P, Chakraborty S, Ponnusamy MP, Lakshmanan I et al., ***Biochimica et biophysica acta***. 2011; 1815(2):224-40. PMID: 21277939.
6. Mukhopadhyay P, Lakshmanan I, Ponnusamy MP, Chakraborty S, Jain M et al., ***PloS one***. 2013; 8(2):e54455. PMID: 23408941.

Tumorigenesis is a multistep process, and cancers of epithelial origin have been viewed as a clonal disease. However, the role of the tumor microenvironment in cancer progression was first proposed by Bissell and Radisky (Bissell MJ, Radisky D 2001 PMID: 11900251) that underscores the need to study each component of the stroma. Evidence suggests that the apparent rarity of stem cell-like cells of human lymphomas that form tumors in immunodeficient mice might simply be consequence of mismatch(es) between the human cells and the mouse microenvironment (Clarke *et al*., 2006 PMID:16990346; Kelly *et al*., 2007 PMID:17641192). Therefore, we employed an orthotopic syngeneic murine model Clone 66 (Dexter *et al*., 1978) that mimics the growth of human breast cancer cells in immune intact individuals and metastasizes to distant organs (Murphy *et al*., 2002 PMID:12498385). The present study was devised to determine if breast cancer cells that met the definition of putative CSC markers (SP, $CD44^{high}CD24^{low/neg}$, $ALDH1^{+}$, $CD34^{high}$, $CD133^{high}$, $CD49f^{high}$ and others), based on expression of different specific stem cell markers, were functionally homogeneous or heterogeneous, based not only on phenotype but also on *in vitro* assays of stem cells (sphere formation) or limiting cell dilution analyses of *in vivo* tumorigenesis. We have demonstrated that Clone 66 murine breast cancer cells that express stem cell phenotypes are heterogeneous and exhibit different functional properties (*Mukhopadhyay et al., 2013* PMCID: PMC3827106). We demonstrated that supposedly homogenous cell populations exhibit cell-to-cell differences. In addition, the coordinated interaction of epithelial cells with their stroma is fundamental to controlling their growth and differentiation in normal and pathological situations (Donjacour AA, Cunha GR 1991 PMID: 1672086). We have showed that tumorigenic potential of stem and non-stem populations was similar when 6000 cells were transplanted suggesting that the differences of tumorigenic potential exist in soluble factor(s) and/or cytokine/chemokine production. I served as the primary investigator on all of these studies.

1. Mukhopadhyay P*, McGuire TR, Farrell T, Murphy B, Sharp JG. Murine Cl66 orthotopic breast cancer cells with stem cell phenotypes exhibit heterogeneity of in vitro agar colony and tumor sphere formation; *and in vivo* tumor growth (Abstract #171). 9th biennial meeting on Mechanisms & Models of Cancer, Cold Spring Harbor Laboratory, New York. August 14-18, 2012 (Poster presentation).
2. Mukhopadhyay P, Farrell T, Sharma G, McGuire TR, O'Kane B, Sharp JG. PloS one. 2013; 8(11):e78725. PMID: 24265713.

The ABC family transporter ABCC11 has been demonstrated to be associated with drug resistance in several cancers and showed worse disease free survival in patients with breast cancer. Currently, we have demonstrated that

overexpression of ABCC1 correlates with breast cancer patients' survival. In other study, our lab showed that the LHML (ligation of left and median hepatic bile duct with gallbladder removal) model was identified to be the most feasible model to study the effect of long-term obstructive jaundice (Aoki *et al.,* 2016 PMID: 27916350). Other important study in our laboratory showed that S1P-enhanced inflammatory cell recruitment leads to rapid and scarless wound healing. I served as co-investigator in all of these studies. Yamada and colleagues previously reported that patients with ABCC11^{pos} tumors have worse breast cancer survival (Yamada *et al*., 2013 PMID: 23288347) [8] [8]. When gene expression of nasopharyngeal carcinoma stem cell was screened, ABCC11 was one of the genes that were overexpressed (Lun *et al*., 2012, PMID: 23285037), indicating that ABCC11 might be involved in cancer stem cell expansion and/or maintenance. Here, we are showing that ABCC11 is associated with the ALDH1^{pos} cell expansion. The significance of this study is that it not only identify the mechanism(s) of ABCC11 mediated expansion of ALDH1^{pos} cell, but also potentially acquire knowledge that lead to the improvement of the breast cancer patients' survival. I served as the primary investigator on this study.

1. Yamada A, Mukhopadhyay P*, Nagahashi M, Aoyagi T, Huang WC, *et al*., Overexpression of ABCC1, but not ABCB1, correlates with breast cancer patients' survival (Abstract #34). VCU Massey Cancer Center Research Retreat, Richmond, VA. May 23rd, 2014 (Poster presentation).
2. Mukhopadhyay P, Ramanathan R, Takabe K. ***Breast cancer management***. 2015; 4(5):241-244. PMID: 27293484.
3. Aoki H*, Raza A, Mukhopadhyay P, Terracina KP, Barnett CC, Spiegel S, Takabe K. Role of Sphingosine Kinase 1 of the Host in the Pancreatic Cancer Peritoneal Carcinomatosis. 2015. 11th annual Academic Surgical Congress. Abstract ID: ASC20151113.
4. Aoki H*, Aoki M, Mukhopadhyay P, Barnett C, Spiegel S, Takabe K. Inflammation Induced by Sphingosine Kinase of the Host Aggravates Pancreatic Cancer Peritoneal Carcinomatosis. 2016. Annals of Surgical Oncology 23, S178.
5. Aoki H*, Aoki M, Katsuta E, Fernandez LJ, Mukhopadhyay P, Yang J, Zhou H, Spiegel S, Takabe K. Obstructive jaundice aggravate pancreatic cancer *via* Sphingosine-1-Phosphate receptors. 2016. 11th annual Academic Surgical Congress. Abstract ID: ASC20161278.
6. Aoki H, Aoki M, Yang J, Katsuta E, Mukhopadhyay P *et al*., ***The Journal of surgical research***. 2016; 206(1):118-125. PMID: 27916350.
7. Aoki M, Aoki H, Mukhopadhyay P, Katsuta E, Takabe K (2018). ***J Invest Dermatol***. PMID: 30367873.
8. Aoki M, Aoki H, Mukhopadhyay P, Yamamoto H, Matsumoto NM, et al. ***International Journal of Molecular Sciences*** (2019). 10 July, doi_ 10.3390_ijms20143381.

* Presenting author

Complete List of Published Work in MyBibliography

http://www.ncbi.nlm.nih.gov/sites/myncbi/1TON8Xx4O-GAq/bibliography/48147929/public/?sort=date&direction=ascending

D. Research Support

- Current:

None

-Past Research supoort

1. 12/1992 – 12/1994: Junior Research Fellowship (Department of Biotechnology, Govt. of India, India).

2. 12/1994 – 10/1995: Senior Research Fellowship (Department of Biotechnology, Govt. of India, India).

3. 09/1998 – 09/2002: Senior Research Fellowship (Indian Council of Medical Research, Govt. of India, India).

Other support For Dr. Mukhopadhyay:

-None

8-Spetial Reporting Requirements

N/A